AXENIC CULTIVATION OF THE BRINE SHRIMP ARTEMIA SALINA

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In a previous paper (Provasoli, Shiraishi and Lance, 1959) we have added to Gibor's (1956) observations that related species of algal flagellates may be either good or bad food for *Artemia*. This idiosyncrasy may depend upon nutritional deficiencies in the algal food, on toxic metabolic products, or even upon some nutrient in excess. One way to attack this ecological problem is to grow *Artemia* on a non-living medium as a step toward a chemically defined medium and, finally, identification of all its nutritional requirements.

The present paper concerns the first stage, *i.e.*, the growth of *Artemia* on a non-living complex medium.

Materials and Methods

The amphigonic American race of *Artemia salina* was employed. Utah brine shrimp eggs (Aquarium Stock Co. Inc., 31 Warren Street, New York 7, New York) proved more satisfactory than other samples tried in respect to percentage of hatching and speed of development.

Sterilization of the eggs. Durable eggs of *Artemia* obtained commercially always contain many dead dried eggs whose chorion is cracked. These eggs are lighter and cannot be disinfected as rapidly as the intact viable ones and should be eliminated at the onset to avoid infections from the inoculum. The technique of disinfection is a modification of the one employed by Gibor (1956).

The dead eggs, being lighter than the viable ones, are eliminated by the flotations in sea water. The eggs are disinfected in screw cap tubes for 10 minutes in Merthiolate solution (1 : 1000 in H_2O) + 0.2 ml.% of a 10% solution of Aerosol OT. to improve wettability. The disinfectant is decanted and the eggs washed in three baths of sterile sea water. The egg slurry is distributed into several tubes of a sterility-test medium (*STP*, Table I) and allowed to hatch 2–3 days at 22–26° C.). Contamination generally shows in 2–4 days. The new-born nauplii develop to second metanauplii at the expense of the reserves of yolk within 4–7 days; the third metanauplii, if not fed, die.

The metanauplii are transferred into a nutrient medium 1–2 days after the first nauplii have hatched, to secure a more uniform inoculum in respect to age; hatching is spread over several days.

The growing larvae consume the particulate food rapidly and must be transferred approximately every 6–8 days, especially after the fourth stage. Transfers are made with Pasteur pipettes connected to a mouthpiece by rubber tubing, to allow a clear view while fishing the larval forms. The later larval stages and the young adults defy suction unless sucked head first, while swimming toward the tip of the pipette. To avoid air-borne infection we used a transfer hood (top and back glass.

TABLE I

STP medium

Sea water	80 ml.
H_2O	15 ml.
Soil extract	5 ml.
$NaNO_3$	20 mg.
K_2HPO_4	1 mg.
Na H glutamate	50 mg.
Glycine	10 mg.
DL-Alanine	10 mg.
Vitamin No. 8A*	0.1 ml.
Trypticase (B.B.L.)	20 mg.
Yeast autolysate (Albimi)	20 mg.
Sucrose	0.1 g.
pH 7.5	

* See Table 4, p. 408 in Provasoli *et al.* (1957).

open in front) with a "Letheray" germicidal UV lamp (see description and figure in Provasoli, Shiraishi and Lance, 1959).

Preparation of media. The following medium (Table II) allows growth to adulthood.

TABLE II

Complete medium

STP (1)	100 ml.
Cholesterol (2)	200 μg.
Dehydrated liver infusion No. L 25 (3)	100 mg.
Trypticase (B.B.L.)	300 mg.
Alkaline-hydrolysed nucleic acid (4)	40 mg.
Acid-hydrolysed DNA (5)	10 mg.
Sucrose	200 mg.
Vitamins mix Art. II (6)	1 ml.
Paramecium factor (7)	5 mg.
Glutathione (8)	30 mg.
Ascorbic acid (8)	3 mg.
Horse serum (aseptic)	5 ml.
Rice starch (9)	500 mg.
pH 7.5	

(1) See Table I.
(2) Dissolved in ethanol.
(3) Oxoid Ltd., England.
(4) Yeast RNA brought to pH 9.0 with NaOH and steamed for one hour.
(5) Herring DNA brought to pH 1.5–2.5 with H_2SO_4 and steamed for two hours.
(6) Vitamins mix Art. II

Thiamine HCl	10 mg. %
Biotin	0.5 mg. %
Folic acid	7 mg. %
Nicotinic acid	50 mg. %
Choline	500 mg. %
Ca pantothenate	70 mg. %
Pyridoxine HCl	8 mg. %
Carnitine	20 mg. %
Riboflavin	0.1 mg. %

(7) Paramecium factor was kindly supplied by Dr. D. M. Lilly (1 part dried yeast cells + 1 part H_2O, autolyzed at 58–60° C. for two hours; the particles are centrifuged and the supernatant vacuum-dried; to obtain a fairly good suspension, bring to a boil.
(8) Fresh solutions of glutathione and ascorbic acid, sterilized by glass-filtration, are added before inoculation.

At first the starch was added to autoclaved media as a slurry. Rice-starch powder is mixed with glass beads (200 μ diameter-Superbrite type 100, Minnesota Mining and Manuf. Co.) and sterilized for two hours at 180° C.; sterile water is added to form a slurry.

We found later that *Artemia* ingests cooked starch equally well; this eliminated one aseptic addition. To prevent the starch from forming a semi-solid mass during sterilization, 500 mg.% of starch powder is added to the complete medium (minus other aseptic additions) before sterilization; the medium is brought to a boil while being stirred vigorously with a glass rod or on a heating plate equipped with a magnetic stirrer. The starch, on boiling, forms small floccules which become larger upon cooling but remain acceptable to *Artemia*. The medium is then tubed and autoclaved.

Miscellaneous preparations:

a) The cholesterol is generally added as an alcohol solution. It has also been employed absorbed on cellulose powder ("Celluflour," Turtox) following the techniques of Singh and Brown (1957) (1 mg. of cholesterol dissolved in 10 ml. ether is mixed with 200 mg. Celluflour; let dry, then added to media before autoclaving).

b) Fatty acids absorbed on starch: 980 mg. of the following fatty acid mixture (proportions of House and Barlow, 1956) palmitic acid 200 mg. + stearic acid 100 mg. + oleic acid 480 mg. + linoleic acid 150 mg. + linolenic 50 mg. are dissolved in ether and mixed with 10 g. of starch; after evaporation of the ether, the powder is sterilized in ethylene oxide for twelve hours; the powder is made aseptically into a slurry with water, and a solution of NaOH or $Ca(OH)_2$ added to neutralize the fatty acids.

c) Albumen (bovine) fraction V (N.B.C.) is dissolved in sea water, glass-filter-sterilized, and added aseptically.

Fraction V was employed also as a carrier of cholesterol and the fatty acids mixture. Sterile solutions of fraction V and cholesterol (dissolved in ethanol) are mixed and added after autoclaving. Two ml. of a sterile 8% solution of Fraction V were mixed aseptically with 2 ml. of an autoclaved fatty acid mixture (dissolve in 50 ml. of H_2O, 1 ml. of concentrated NaOH, stearic acid 24 mg., palmitic acid 54 mg., linoleic acid 36 mg., linolenic acid 12 mg., oleic acid 0.12 ml., adjust to pH 8.0 with HCl, autoclave).

Interpretation of Results

To compare the nutritive value of different foods and supplements we needed to recognize the various larval forms by characters easily visible by inspection of the tubes with a hand lens. We could not resort to the fine external morphological

characters employed by Heath (1924) to divide in 13–15 instars the development of *Artemia* from egg to adult; these differences can be evaluated only with a microscope. We decided therefore to employ, slightly modified, the arbitrary nomenclature of Barigozzi (1939) which divides the life-cycle in stages of development clearly distinguishable with the hand lens.

The nauplius is small, roundish, yellow-pink (first instar of Heath). Metanauplius I is small, triangular, yellowish (second instar of Heath). Metanauplius II is similar but bigger (third instar of Heath).

Metanauplius III. "Small" III T-shaped, longer, thin, shows a visible segmentation in the upper thoracic region (fourth instar of Heath); "big" III (1.5 mm.); the first 3–5 thoracic limbs are well developed but do not move well (fifth instar of Heath). The first three metanauplii stages are easily distinguished from the other stages, even with the naked eye, by their jerky swimming: at these stages propulsion depends upon the characteristic backward and forward paddle-like movement of the long second antennae.

Metanauplius IV. "Small" IV (2 mm.). The first thoracic limbs are now moving well and the movement of *Artemia* is a combination of jerks and swimming in circles (sixth instar of Heath); "medium" IV (2.5 mm.) swims on its back gracefully in circles; most of the thoracic limbs are fully articulated and move rhythmically like rippling waves (seventh instar of Heath); "big" IV (3.5 mm.) bigger in size, abdomen longer and slender, the second antennae smaller than the limbs and lying parallel to the head (eighth instar of Heath).

"Juveniles" (5–7 mm.) have stalked eyes, the abdomen elongates and becomes segmented, at the tip of the abdomen the furca becomes evident; they have a slender appearance and resemble adults but are much shorter (ninth-eleventh instars of Heath).

Adults (7–10 mm.). The males have long claspers (modified overgrown second antennae); the females have a slender head and a conspicuous egg-pouch right below the last pair of limbs (twelfth-fifteenth instars of Heath).

We record twice a week the stage reached in each tube. As in many insects, some phases of the life-cycle of *Artemia* seem more critical than others: the transition from the third to the fourth stage and the one between "big IV" to "juveniles." In general, a good way to evaluate the effect of the different supplements is to compare (a) the days required to reach the "small IV" metanauplius, (b) the days needed to reach adulthood, and, if they do not become adults, (c) the stage reached at death and days elapsed since birth.

We generally inoculate 5–8 larvae per tube. Not all develop into adults even in the best media although in these media most do. Quite often, especially from the III metanauplius onward, "black disease" develops: black spots, consisting of fine melanin granules, develop at the lobes of the phyllopodia, especially on the dactylopodite. The incidence of black-spotted individuals is sporadic and could not be correlated with any particular nutritional deficiency; it might be simply a difficulty in molting, *i.e.*, left-over parts of the previous cuticle may impede normal development. We cannot say how much these spots affect normal growth and if they are harmful; in complete media (Table II) the adults often had black spots from the IV metanauplius on, yet they could become adults. Black disease is often common; until its causes and effect on the health of the larvae are known it

is impossible to use accurately the percentage survival of a mixed population of normal and black-spotted larvae as an index of the nutritional status.

Results

a) Particulates

Artemia is a voracious particle-feeder as we amply observed when rearing them on living flagellates. We thought that this behavior could be exploited to increase the ratio of ingestion (drinking) of the nutrients added as solutes, because little absorption can be expected by an arthropod except from the middle intestine, the rest of the body being clad in chitin. An ideal situation would be to have a nutritionally inert attractive particle and to supply nutrients as solutes, thus permitting the application of the usual microbiological techniques for replacing complex organic substances with chemically defined components. We tried a variety of particles, many of them nutritionally rich, because we did not know whether the organisms could withstand a medium rich enough in solutes to support their growth. Early experiments had shown that 0.6% Trypticase inhibits *Artemia* and that *Tigriopus* is even more sensitive to organic solutes. The following compounds were ground fine (between 5–20 μ) in a colloidal mill, sterilized by dry heat, and added aseptically as water suspensions to the liquid part of the medium. The liquid (STP, Table I, + a vitamin mix, and 100 mg.% of "Oxoid" liver infusion, no. L 25) employed at the time is nutritionally deficient and, in the absence of particles, *Artemia* grow only up to medium-sized III metanauplii. Additions of 150 mg.% of particulate blood fibrin, yeast cells, corn protein, lactalbumen, Cerophyll, casein, Celluflour, and rice polishings were ineffective, while fish meal or gluten permitted reaching the IV in 23–25 days; rice starch did so in 35–40 days. Rice starch was selected because it is, if digested, mainly a carbon source. It is almost devoid of impurities of other important nutrilites (as is gluten) and therefore offers the possibility of defining requirements for amino acids, proteins, fats, and vitamins.

Larval forms of *Artemia* are voracious: suspended particles are quickly transformed into fecal pellets. We therefore raised the particulate starch to 0.5% and kept it suspended as much as possible by shaking and homogenizing the medium twice daily. Later on, when solutions allowed growth beyond the third metanauplius, we had to transfer the growing metanauplii every 7 days to a fresh medium and to increase the volume of the medium from 5 ml. to 10 ml. Five ml. of medium in 20 × 125 mm. tubes are better for the growth of young larvae (up to the early stages of "small IV") because such larvae swim poorly, feed mostly at the bottom, and need a medium well aerated by a large surface exposed to the air. Later stages are continuously swimming and stirring the medium. Perhaps the later larval stages would grow faster if the media were changed even more often, but this requires much patience and increases contamination (see "Methods"). It was found later that the starch can be added before sterilization if it is precooked while stirring the medium (see "Preparation of Media"); the resulting floccules are still ingested, and remain more easily in suspension.

When we found a medium allowing growth to adulthood (Table II), we reinvestigated the necessity of particles. The liquid part of the medium (excepting the heat-sensitive components of the medium) was autoclaved, filtered through

paper, then glass-filter-sterilized, and dispensed aseptically into tubes. Sterile solutions of glutathione, ascorbic acid, and the serum were added last.

This medium is clear and devoid of visible particles. The nauplii of *Artemia* in this medium reached at best the stage of "big III" metanauplii. In the same batch of medium to which was added aseptically a sterile starch slurry, *Artemia* reached adulthood. A similar experiment was done recently but with another medium allowing growth to adulthood; again the absence of particles prevented growth beyond the third metanauplius.

b) Solutes

Trypticase and nucleic acids. In preliminary experiments it was soon discovered that addition of Trypticase (0.3%) and nucleic acids to the liver extract speeded growth greatly; the IV metanauplius stage was reached in 12–19 days but growth stopped at medium-size metanauplii. Whole blood (1 ml./100) and a suspension of red blood cells, as substitutes for the starch particles, did not speed growth or allow a more advanced stage; yeast cells (autoclaved) were inhibitory.

Vitamins. Since the level of the vitamin mixture initially used (Table I) was far below the levels for insects, we suspected that the medium was mainly deficient in vitamins. Tentatively, we chose concentrations and ratios similar to those employed for insects, but at the lower limits because in earlier experiments we found that cholesterol was already inhibitory at values which are low for many insects. Biotin, pyridoxine, folic acid, nicotinic acid, and choline, added singly and in combinations, either affected general vitality (*i.e.*, vigorous swimming), speeded the time required for reaching the fourth metanauplius stage, or permitted growth up to "very big" IV metanauplii. Therefore we designed richer and more complete vitamin mixtures (see latest in Table II). To see whether some vitamins were present in suboptimal or toxic concentrations, we removed singly each vitamin and added it at different concentrations. Thiamine and folic acid proved limiting, indicating that Trypticase, liver extract, and serum at the levels employed in the complete medium (Table II) are inadequate sources of these vitamins for *Artemia*. Adulthood was reached in the complete medium and no inhibitions were found up to the following maximal concentrations tried (wt./100 ml. final medium): thiamine 200 μg., biotin 30 μg., folic acid 300 μg., nicotinic acid 1 mg., pantothenic acid 3 mg. Choline did become inhibitory between 3 and 10 mg., pyridoxine between 50 and 100 μg.%, and riboflavin between 0.1 and 1.0 mg.%.

Serum, glutathione, and paramecium factor. Adults were not obtained until horse serum, glutathione, and paramecium factor were added to the medium. Serum alone reduced the time to reach the IV metanauplius from 29 days (no serum) to 19 days (2 ml./100), and to 13 days (4–10 ml./100); increasing the serum allowed growth up to "big" IV metanauplii.

In the absence of serum, 20–40 mg.% of glutathione enabled the metanauplii to reach the IV stage in 16 days and become "small" IV. Paramecium factor alone or in combination with glutathione seems ineffective, but when added to the combination glutathione plus serum, allows adulthood. Under our conditions, only "very big" IV or juveniles were produced by serum + glutathione; this combination was quite effective in speeding growth; the IV metanauplius was reached in 13 days. For production of adults the serum should reach the level of 3 ml./100

or more (up to 10 ml./100); when the serum was below 3 ml./100, depending on the quantities of serum added, only "big," "medium," or "small" IV metanauplii were produced.

Cysteine can substitute for glutathione in eliciting adulthood: 10 mg.% cysteine was as effective as 20–30 mg.% glutathione.

Horse serum can be substituted with a filter-sterilized solution of dried beef serum (Difco).

The active factors present in serum are heat-resistant: both supernatants of the autoclaved horse or Difco beef serum, glass-filter-sterilized and added aseptically, are as effective as serum.

Cholesterol and fatty acids. Some attempts were made to replace serum. Five milliliters of serum supply, *inter alia*, much neutral fat, lecithin, cephalin, and cholesterol. The medium without serum, except for the concentrations brought in possibly by 100 mg. of Oxoid liver, has no fatty acids and only 200 μg.% cholesterol. Some insects require long-chain unsaturated fatty acids (linoleic acid, linolenic acid); all require cholesterol. Early experiments had shown that cholesterol above 500 μg.% becomes inhibitory, but these experiments were done with poor media. In comparable media, linoleic acid was indifferent at 1 mg.% and inhibitory at 10 mg.%. With better media, cholesterol absorbed on Celluflour inhibited at or above 6 mg.% and linolenic acid was inhibitory above 1 mg.% and became rapidly toxic. Neither cholesterol or linolenic acid replaced the serum. The fatty-acid mixture absorbed onto starch powder was indifferent at 2–5 mg.% and quite toxic above. Thinking that the toxicity might be caused by the acidity of the acids, the sterile fatty-acid starch slurry was adjusted with NaOH or $Ca(OH)_2$; the Na salts are far more toxic than the Ca salts.

Cholesterol, or the fatty acid mixed with serum albumen fraction V, also did not replace serum. However, 200 mg.% of fraction V alone allowed normal adults in two months instead of 25 days; growth up to the IV metanauplius was as in serum. Soya lecithin became rapidly toxic; "Gliddex I" (a refined "lecithin" containing 4% lecithin, 29% cephalin, 55% inositol phosphatides, and 4% soybean oil) absorbed either on starch or casein, is well tolerated up to 10–15 mg.%. However neither lecithin, Gliddex I or other "lecithins" replaced serum.

Discussion

While *Artemia* can be grown without living food and tolerates concentrations of solutes sufficient to permit development to adults, it does not grow wholly on solutes. The particles are needed to stimulate filter-feeding, thus allowing ingestion of enough nutrient solutes for a normal growth rate.

The studies of Croghan (1958a, 1958b) on osmotic regulation in *Artemia* show: *a*) that the cuticle of the branchiae (metepipodites) of the first 10 pairs of the phyllopods is the only part of the external body cuticle that is appreciably permeable in the adults; in the young larvae, where the phyllopods have not yet developed, the neck organ is the permeable region; *b*) the branchiae are the site of active NaCl excretion; *c*) the gut epithelium controls internal water balance by water uptake; *d*) *Artemia* continues to swallow the medium even when devoid of particles. The swallowing behavior of the adults was indicated by the red coloration of the gut walls a few hours after *Artemia* were put into a filtered phenol red solution.

Our experiments show that the rate of swallowing of a particle-free medium is probably very low in the early metanauplii stages—certainly insufficient to provide enough nutrient solutes for growth. Particles stimulate swallowing. Since all the nutrients in our media are in solution, Trager's (1936) conclusion, based on *Aedes aegypti,* that solutes are utilized for growth, applies to Crustacea. Although other invertebrates, like some ciliates, can be grown on solutes, perhaps some phagotrophs, including Crustacea, living in oligotrophic waters, may be obligate or partial phagotrophs because particulate feeding, besides increasing the ingestion of nutrient solutes, does not affect their inability to withstand concentrations of organic solutes high enough to support growth (Provasoli, 1956).

The medium allowing growth to adults (Table II) is still quite complex; it offers, however, more possibilities of dissection than the only other known axenic medium for a crustacean—the blood-glucose mixture of Treillard (1924) for *Daphnia.* It was exciting to have started with an inadequate medium because each experiment permitted the demonstration of some nutritional needs, some of them reflecting obvious requirements, as was the effect of Trypticase and nucleic acids. It is remarkable that thiamine and folic acid were required even in the presence of Trypticase and liver extract which are ordinarily adequate sources of these vitamins. Glutathione is also required and was replaceable by cysteine. So far only another arthropod, the mosquito *Aedes aegyptii,* requires glutathione (Singh and Brown, 1957), even in the presence of adequate cysteine. Interestingly, the "feeding reaction" of *Hydra* is controlled by glutathione which acts as a specific "feeding hormone"—it is not replaceable by cysteine, ascorbic acid or other donors of SH groups (Loomis, 1955).

Serum is required for the full development of adults. The active components of serum are heat-stable. We could not replace serum with mixtures of fats and cholesterol. These results are only indicative: lack of effect may be due to toxicities of some components of the fatty acid mixture or failure to avoid toxicity by presenting them to *Artemia* on the proper fat carrier.

However, if the toxicity of fatty acids is the cause, it might explain some of the nutritional idiosyncrasies found previously(Provasoli, Shiraishi and Lance, 1959). Utilization of flagellates as food may depend as much upon their providing *Artemia* with all the nutrilites needed as with their lacking toxic substances and vice versa when they are not utilized as food.

For *Artemia* we found that especially in the *Chlorophyta* several species, even strains, were inadequate as food while others were not. Chlorophytes are known to produce toxic unsaturated fatty acids such as chlorellin (Spoehr *et al.,* 1949). Indeed this might well be a characteristic of the *Chlorophyta.* Proctor (1957) found that *Haematococcus* is particularly sensitive to palmitic, oleic, and linoleic acids, and also to substances produced by cultures of *Chlamydomonas reinhardii.* The substances produced by *Chlamydomonas* are steam-distillable and fat-like, quite probably a mixture of unsaturated fatty acids. The accumulation of oil droplets is readily observed in different species of *Polytoma,* the colorless counterpart of *Chlamydomonas.* During the logarithmic phase the cells are full of paramylum granules, but as they pass the peak of growth the starch is replaced in great part by fat droplets. This might explain the toxicity of aged cultures of *Chlorella* to *Daphnia magna* found by Ryther (1954).

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Summary

1. *Artemia salina* can be grown aseptically to adults in a non-living medium.

2. The components of the medium are: sea water, Trypticase, liver infusion, hydrolysed RNA and DNA, serum, sucrose, cholesterol, paramecium factor, glutathione, a mixture of B vitamins, and starch particles.

3. Glutathione, thiamine, and folic acid were found essential even in the presence of Trypticase and serum. Glutathione can be replaced by cysteine. Horse or beef serum (Difco) supply unidentified heat-stable nutrients. Cholesterol and mixtures of fatty acids become rapidly toxic, and do not replace serum.

4. *Artemia* is a voracious particle feeder and transforms the starch particles rapidly into fecal pellets. In the absence of starch particles, the liquid part of the medium, though containing all the nutrients, supports growth only to the third-stage metanauplii. This indicates that the rate of ingestion (swallowing) of liquids is too low to support continuous growth and that the particles are necessary to increase the swallowing reaction.

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